ISSN (print) 0093-4666 ISSN (online) 2154-8889

MYCOTAXON

http://dx.doi.org/10.5248/118.17

Volume 118, pp. 17–26 *October–December 2011*

Discrimination of *Gigaspora* species by PCR specific primers and phylogenetic analysis

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Abstract — Species of arbuscular mycorrhizal fungi (AMF) are usually identified by the morphological characteristics of their spores. However, considering the difficulties in diagnosing their taxa, the construction of species-specific primers has been proposed as an identification alternative. In this paper the problem of distinguishing different *Gigaspora* species with slight morphological differences was solved using species-specific primers and SSU and LSU rDNA sequence analyses of 18 AM fungal isolates comprising seven species. Neighbor joining, maximum parsimony, and maximum likelihood analyses were performed to evaluate the phylogenetic affiliation of the isolates, and a new reverse PCR primer (ALB1) specific for *Gigaspora albida* was designed and tested with 11 *Gigaspora* isolates (four species). The results confirmed misidentification of '*G. albida*' FL 927 and '*G. margarita*' BR 444 and supported referring FL 927 to *G. rosea* and BR 444 to *G. albida*.

Key words — phylogeny, ribosomal sequences, *Glomeromycota*

Introduction

There are approximately 220 species of arbuscular mycorrhizal fungi (AMF) (Stockinger et al. 2010). Morphological differences in spore structure are usually used to distinguish between individual species, but this requires a great deal of experience (Morton 1993, Bentivenga & Morton 1994). More practical methods are needed, so that AMF can be identified directly not only in the rhizosphere, but also after host root colonization. DNA analytical methods involving electrophoretic profiles (Wyss & Bonfante 1993), sequence comparisons (Daniell et al. 2001, Husband et al. 2002, Mummey & Rillig 2007, Stukenbrock & Rosendahl 2005), PCR-DGGE (Souza et al. 2004), species-

specific primers (Gamper & Leuchtmann 2007, Geue & Hock 2004, Lanfranco et al. 1995, 1999, 2001, Millner et al. 1998, 2001a, 2001b, Redecker 2000), and DNA barcodes (Stockinger et al. 2010) are being developed that would allow identification of individual AMF species in an ecological context. For example, species-specific PCR primers could be used to discriminate morphologically similar species or even to identify species within colonized roots. However, to date only a small number of species-specific probes has been developed.

After Oehl et al. (2008) divided the *Gigasporaceae* into four families and six genera, Morton & Msiska (2010) proposed dividing *Gigasporaceae* into just three genera —*Gigaspora*, *Racocetra, Scutellospora. Gigaspora* has five species (Bentivenga & Morton 1995) in which the morphological variation is low (Bago et al. 1998, Souza et al. 2004, Lanfranco et al. 2001), and only diameter, color and spore wall thickness of glomerospores have been used for species identification (Bentivenga & Morton 1995). Species-specific primers designed for *G. margarita* (Lanfranco et al. 1999) and *G. rosea* (Lanfranco et al. 2001) have shown to be effective in solving such morphological conflicts. In this study we show how a species-specific PCR primer constructed for *G. albida* helps solve problems associated with discriminating *Gigaspora* species and evaluate *Gigaspora* phylogenetically through SSU and LSU rDNA analyses.

Materials & methods

AM fungi

Eleven reference isolates (Table 1) representing *Gigaspora* propagated in pot cultures were selected.

Intracellular bacteria

Presence of intracellular bacteria was assessed with the Live/Dead *Bac*-Light bacterial viability kit (Molecular Probes, Inc., Eugene, OR, USA) following Bianciotto et al. (1996).

DNA extraction

Ten to 50 spores were washed in distilled water, sonicated 3–4 times, crushed in 50–100 µl of 1x REDTaq PCR Reaction Buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$ and 0.01% gelatin) (Sigma-Aldrich, Milan, Italy), and centrifuged at 1000 RPM for 2 min. The supernatant containing the DNA was incubated at 95°C for 13 min. After extraction, the DNA was stored at –20°C.

Design of PCR primers, amplification and sequencing

A new species-specific reverse PCR primer ALB1 (5'-CCCAACTAAAAATACTTCAGTC-3') was designed for *G. albida* based on the GenBank ITS sequence AJ239118 and combined with GiITS1 (Lanfranco et al. 1999) to create a 385 bp fragment. For *G. margarita* and *G. rosea,* combinations of primers GiITS1+GiITS2 (Lanfranco et al. 1999) and GiITS1-GiR3 (Lanfranco et al. 2001) were used, respectively.

Table 1. *Glomeromycota* isolates used in PCR amplification tests with species-specific primers and phylogenetic analysis.

Species[a]	Isolate	Origin	Source[b]
Gigaspora albida N.C. Schenck & G.S. Sm.	BR 601	Brazil	INVAM
G. albida	FL 927	USA	UFPE
G.decipiens I.R. Hall & L.K. Abbott	BEG 45	Australia	BEG
G. gigantea (T.H. Nicolson & Gerd.) Gerd. & Trappe	MN 414D	USA	INVAM
G. gigantea	NC 150	USA	INVAM
G. gigantea	WV 932	USA	INVAM
G. gigantea	NC 199A	USA	INVAM
G. margarita W.N. Becker & I.R. Hall	MAFF 52	Japan	MAFF
G. margarita	MAFF 54	Japan	MAFF
G. margarita	BEG 34	New Zealand	BEG/UNITO
G. margarita	BR 444	Brazil	INVAM
G. rosea T.H. Nicolson & N.C. Schenck	UT 102	USA	INVAM
G. rosea	MAFF 62	Japan	MAFF
G. rosea	BR 151 A	Brazil	INVAM
G. rosea	DAOM 194757	NA	NA
Scutellospora calospora (T.H. Nicolson & Gerd.) C. Walker & F.E. Sanders	BEG 32	UK	BEG
S. calospora	HDAM-3	na	na
Scutellospora sp.	W 3485	UK	C. Walker

[a] Species used in the test with species-specific primers (in bold). [b] INVAM: International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi, USA; UFPE: Universidade Federal de Pernambuco, Departamento de Micologia, Brasil; MAFF: Ministry of Agriculture, Forest and Fisheries, Japan; BEG European Bank of Glomales, France; UNITO: Università di Torino, Dipartimento di Biologia Vegetale, Italy; na: information not available.

The 28G1 and 28G2 primers (Silva et al. 2006) were used to amplify the partial LSU rDNA region. NS1, NS2, NS3, NS4, NS5 and NS8 were used to obtain the SSU rDNA amplicons (White et al. 1990).

PCR reactions were carried out in a volume of 50 µl, containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$, 0.01% gelatin, 200 µM each dNTPs, 1 µM of each primer and 2 units of REDTaq™ DNA polymerase (SIGMA) (Sigma-Aldrich, Milan, Italy). Cycling parameters were 45 s at 94°C, 1 min at 55°C, 1 min at 72°C for 40 cycles, with a final elongation of 7 min at 72°C followed the last cycle. The amplicons were visualized on 1.5% agarose gel with ethidium bromide. The amplified products for SSU and LSU rDNA were purified with a QIAquick kit (Qiagen S.p.A., Milan, Italy) following the manufacturer's instruction and sequenced (accession numbers are listed in Table 1). Sequencing was made by GeneLab (Roma, Italy).

Sequence alignment and phylogenetic analysis

Prior to phylogenetic analysis, a BLASTn query of the National Center for Biotechnology information databases demonstrated that sequences obtained from AMF species were affiliated with the genus *Gigaspora*.

Sequences of 15 *Gigaspora* and 11 *Scutellospora* isolates (some obtained from GenBank) were selected to align SSU and LSU rDNA (Table 1) using Clustal X (Larkin et al. 2007) and edited with BioEdit (Hall 1999).

For phylogenetic analyses and tree construction, neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) analysis with 1,000 bootstrap replications were performed using PAUP4 (Phylogenetic Analysis Using Parsimony vers. 4, Swofford 2003). NJ and ML analyses were performed using parameters obtained from ModelTest 3.7 (Posada & Crandall 1998). *Scutellospora calospora* and *Scutellospora* sp. were used as outgroup.

Results

Morphological observations of isolates FL 927 and BR 444

Isolates FL 927 ('*G. albida*') and BR 444 ('*G. margarita*') had morphological characters similar to other *Gigaspora* species: spore wall thickness [9.6–(12.2)–14.4 µm and 12–(13.9)–16.8 µm, respectively] and spore diameter [230–(282)–360 µm and 250–(320)–384 µm, respectively]. However, the '*G. margarita*' BR 444 spores were hyaline/white to yellow and lacked the characteristic green (*G. albida*) or pink (*G. rosea*) coloration observed in reference isolates *G. albida* BR 601 and *G. rosea* UT 102. All isolates of '*G. margarita*' BR 444 and *G. albida* BR 601 contained endobacteria, which were absent in all isolates of *G. rosea* and '*G. albida*' FL 927.

Testing species-specific primers for taxa of *Gigaspora*

Eleven *Gigaspora* isolates were screened for PCR amplification of a short partial nuclear ITS rDNA fragment (385bp) with species-specific primers (Table 1). The primers GiITS1+GiITS2 amplified only the *G. margarita* species, excluding isolate '*G. margarita*' BR 444, which did not amplify at all (Fig. 1A). The combination of the species-specific primer constructed for *G. rosea* (GiR3) with GiITS1 amplified not only the isolates of *G. rosea*, but also '*G. albida*' FL 927 and showed a slight reaction to the DNA from spores of *G. albida* BR 601 and '*G. margarita*' BR 444 (Fig. 1B), while the GiITS1+ALB1 primer pair amplified only *G. albida* BR 601 and '*G. margarita*' BR 444 (Fig. 1C). These results support a misidentification for '*G. albida*' FL 927 and '*G. margarita*' BR 444.

Phylogenetic analysis of *Gigaspora* from SSU and LSU rDNA

In the SSU rDNA phylogenetic analyses (NJ, MP and ML), the genus *Gigaspora* was supported as a monophyletic group with 100% of the bootstrap value (Fig. 2). Three subclades were observed in the genus, the first grouping '*G. margarita*' BR 444 with all isolates of *G. albida* and *G. rosea*. In the second cluster just *G. gigantea* was present and in the last group all isolates of *G. margarita* (except '*G. margarita*' BR 444) appeared together with *G. decipiens*.

In the tree constructed with the LSU rDNA, *Gigaspora* was monophyletic with high bootstrap support (Fig. 3). All isolates of *G. margarita* (except

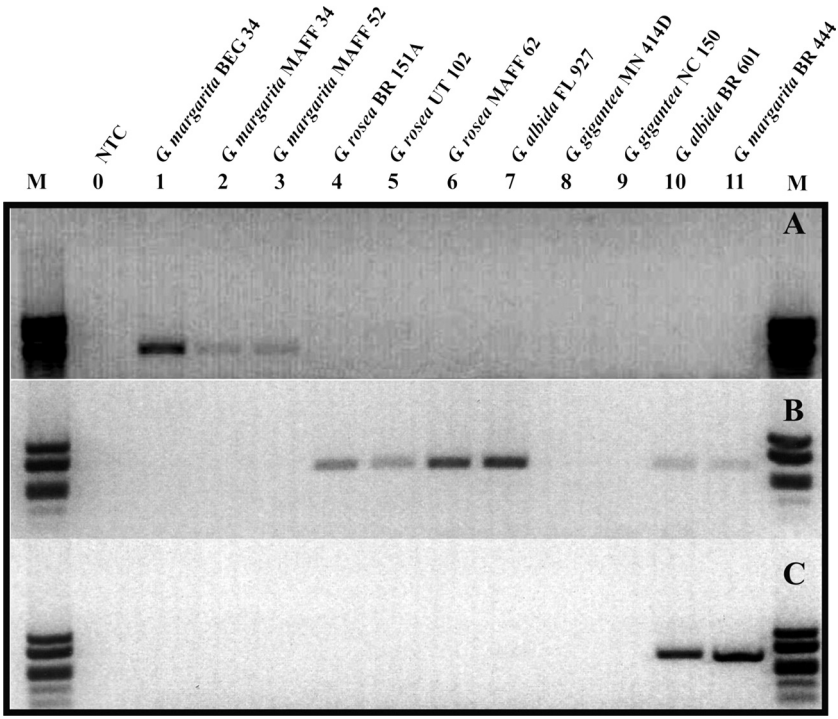

Fig. 1. Agarose gels (1.5%) with PCR products of the *Gigaspora* spp. Different combinations of primers were used: A) GiITS1-GiITS2; B) GiITS1-GiR3; C) GiITS1-ALB1. M = pUC 18 digested with *Hae* III. NTC = no template control.

'*G. margarita*' BR 444) clustered together. Two isolates of *Gigaspora gigantea* grouped in the same clade, *Gigaspora albida* BR 601 and '*Gigaspora margarita*' BR 444 clustered together and *Gigaspora rosea* UT 102 was close to '*Gigaspora albida*' FL 927. Based on SSU and LSU rDNA analyses '*Gigaspora margarita*' BR 444 has been misidentified.

Discussion

Due to the low morphological variation amongst *Gigaspora* species (Bentivenga & Morton 1995), some papers have reported species misidentification (Bago et al. 1998, Souza et al. 2004, Lanfranco et al. 2001). These studies also report the usefulness of molecular approach as an identification tool. Based on sequences of SSU rDNA and isozyme analysis, Bago et al. (1998) divided *Gigaspora* into three subgeneric groups: the first with *G. margarita* and *G. decipiens*, the second with *G. albida* and *G. rosea,* and the third with *G. gigantea.* In this paper these same groups were observed in the phylogeny generated by

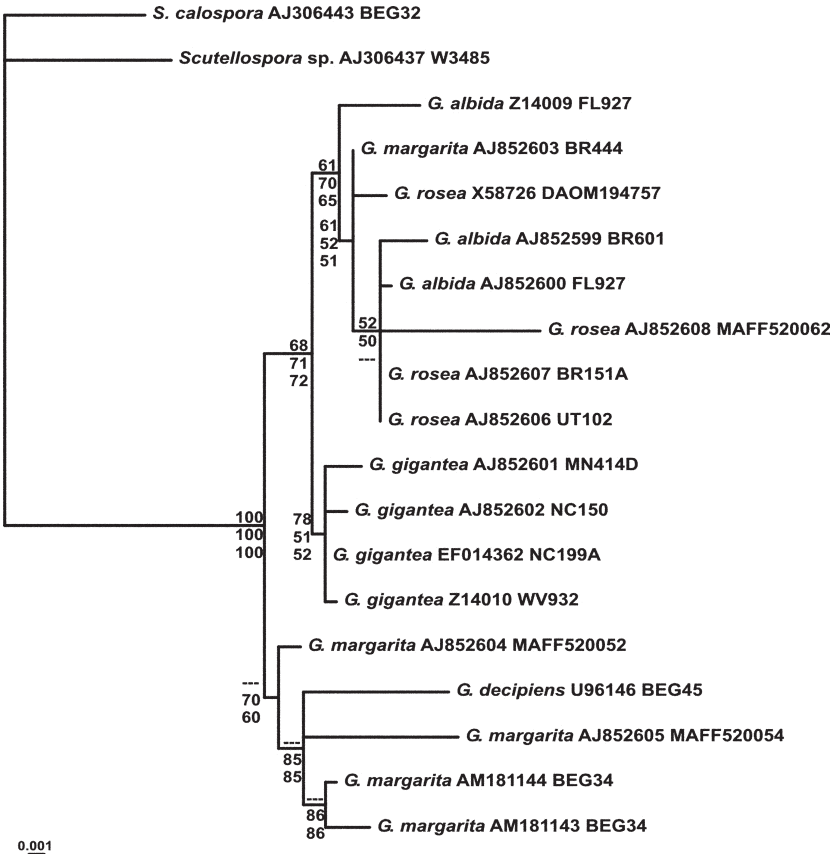

Fig. 2. Phylogenetic reconstruction of the *Gigaspora* obtained from SSU rDNA sequences (~1700 bp). The NJ and ML analyses were performed with HKY85 + I substitution model. Bootstrap values (in %) are from neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) analyses (1000 bootstraps), respectively. Only topologies with bootstrap values of at least 50% are shown. (Consistency Index = 0.88; Retention Index = 0.93).

SSU rDNA, and just '*G. margarita*' BR 444 was out of the correct clade. In the LSU rDNA tree, the isolate BR 444 also grouped together with *G. albida* BR 601, whereas the isolate FL 927 (identified as *G. albida*) was clustered with *G. rosea*. Bago et al. (1998) observed that the isolates BR 444, identified initially as *G. margarita*, and FL 927 (described as *G. albida*), should instead be ascribed to *G. albida* and *G. rosea*, respectively. The PCR amplification tests with species-specific primers in this study also indicated that these two isolates should be reclassified as *G. albida* and *G. rosea*. Nevertheless recently Msiska & Morton

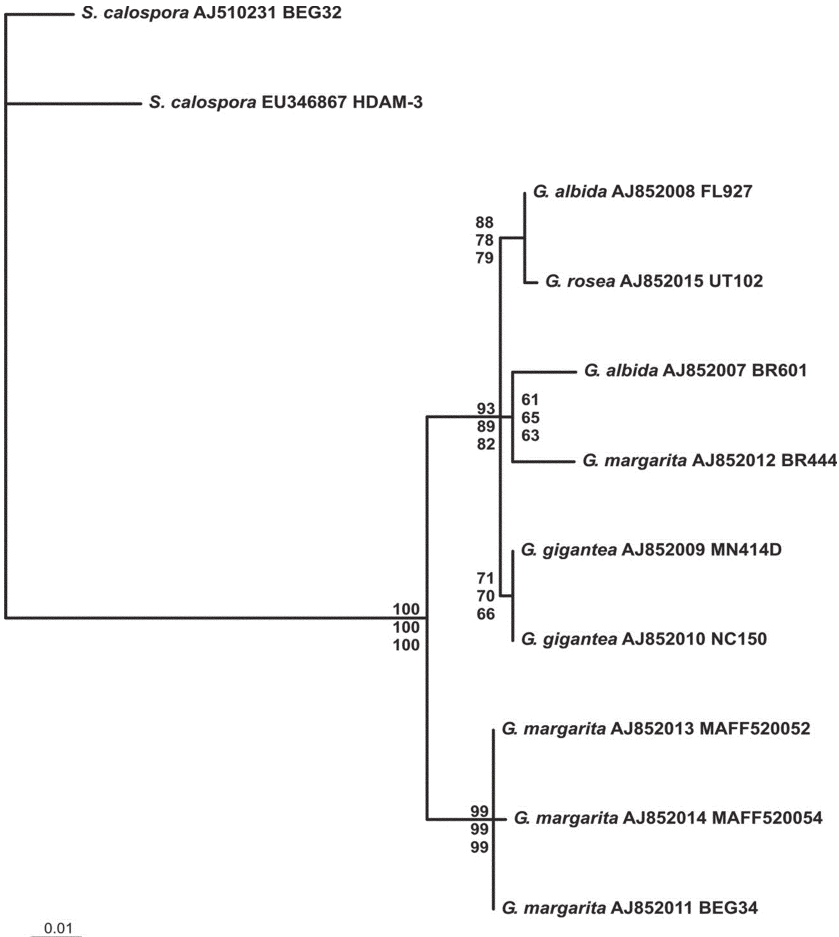

Fig. 3. Phylogenetic reconstruction of the *Gigaspora* obtained from LSU rDNA sequences (~ 450 bp). The NJ and ML analyses were performed with GTR substitution model. Bootstrap values (in %) are from neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) analyses (1000 bootstraps), respectively. Only topologies with bootstrap values of at least 50% are shown. (Consistency Index = 0.93; Retention Index = 0.93).

(2009), based on β-tubulin gene, reported that '*G. margarita*' BR 444 did not group with *G. albida* or *G. rosea*, but with *G. decipiens*.

Using PCR-DGGE analysis, Souza et al. (2004) already observed, as we confirmed here, that the FL 927 isolate, originally identified as *G. albida,* should be ascribed to *G. rosea*, indicating identification problems. Endobacteria are usually observed in glomerospores of *Gigaspora* species, with the exception of

G. rosea isolates. Bacteria were not found in '*Gigaspora albida*' FL 927, further substantiating the need for reclassification.

Van Tuinen et al. (1998) were the first to design specific primers for *Gigaspora* species (*G. rosea*) that amplify a region of the LSU rDNA. Because these authors tested cross-amplification only with species of other AMF genera, it is not possible to establish whether the primers amplify other *Gigaspora* species. Currently there are primers specific for two *Gigaspora* species (*G. margarita* and *G. rosea*); nevertheless, we report that the reverse primer GiR3 (Lanfranco et al. 2001) also amplifies *G. albida* isolates. As Lanfranco et al. (2001) did not use *G. albida* and *G. decipiens* in their analyses with species-specific primers, it was not possible to establish how specific the primers are to *G. rosea* and *G. margarita*. Of the five known *Gigaspora* species (Bentivenga & Morton 1995), only *G. decipiens* was not used in this work.

In summary, our results show that different specific PCR primer pairs and phylogenetic studies can help discriminate among *Gigaspora* species with similar spore morphologies and support the utility of these primers and phylogenetic analysis for solving problems in identifying groups of species in *Gigasporaceae* with a low morphological variation.

Acknowledgements

We are grateful to Dr. Fritz Oehl and Dr. Renata Gomes de Souza for the valuable comments on the manuscript. L.C. Maia acknowledges the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for a research grant. The authors wish to thank Dr. J. Morton and Dr. M. Saito for providing fungal isolates. We are grateful to Dr. David Bousfield for the critical reading of the manuscript and appreciate the corrections by Shaun Pennycook, Nomenclatural Editor, and suggestions by Lorelei L. Norvell, Editor-in-Chief. This research was funded by the Italian National Council of Research (CNR), Cassa di Risparmio di Torino and Centro di Eccellenza per la Biosensoristica Vegetale e Microbica.

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